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Size of the pores created by an electric pulse: Microsecond vs millisecond pulses ☆,☆☆

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ABSTRACT

Here, the sizes of the pores created by square-wave electric pulses with the duration of 100 μs and 2 ms are compared for pulses with the amplitudes close to the threshold of electroporation. Experiments were carried out with three types of cells: mouse hepatoma MH-22A cells, Chinese hamster ovary (CHO) cells, and human erythrocytes. In the case of a short pulse (square-wave with the duration of 100 μs or exponential with the time constant of 22 μs), in the large portion (30–60%) of electroporated (permeable to potassium ions) cells, an electric pulse created only the pores, which were smaller than the molecule of bleomycin (molecular mass of 1450 Da, $r \approx 0.8$ nm) or sucrose (molecular mass of 342.3 Da, radius—0.44–0.52 nm). In the case of a long 2-ms duration pulse, in almost all cells, which were electroporated, there were the pores larger than the molecules of bleomycin and/or sucrose. Kinetics of pore resealing depended on the pulse duration and was faster after the shorter pulse. After a short 100-μs duration pulse, the disappearance of the pores permeable to bleomycin was completed after 6–7 min at 24–26 °C, while after a long 2-ms duration pulse, this process was slower and lasted 15–20 min. Thus, it can be concluded that a short 100-μs duration pulse created smaller pores than the longer 2-ms duration pulse. This could be attributed to the time inadequacy for pores to grow and expand during the pulse, in the case of short pulses.

1. Introduction

Electroporation – modification of the cell membrane permeability by creating nanometer-sized pores by strong electric field – has numerous applications in biology, biotechnology, and medicine [1,2], including nanomedicine approaches [3–5]. The information on the size and number of the pores created by pulses of strong electric field is important. First, to test theoretical considerations, it is important to have the information on the pore population: the minimal and maximal sizes of electrically induced pores and how many of them are created under certain electroporation conditions. The answers to these questions are of interest from the practical standpoint as well. For many applications of cell electroporation, e.g., targeted delivery of drugs [6], genes [7], or nanoparticles [3,8–10] into cells and tissues, it is desirable to know in advance the pores of what size will be created in the cell membrane as it would allow estimating the upper size limit of the molecules that can be introduced into the cells (or could leave the cells). In addition, it would be useful to know whether the size and/or number of pores can be adjusted by varying the parameters of an electric treatment (pulse duration, amplitude, shape, number of pulses, etc.).

There are numerous studies in which the size of the pores created in the cell membrane by pulses of strong electric field has been estimated, both theoretically [11–14] and experimentally [15,15–22]. According to theoretical modeling, the pulses of nanosecond-duration should create smaller pores than the pulses of micro-millisecond duration [11]. At the same time, the number of pores created by nanosecond-duration pulses should be by 2–3 orders of magnitude larger than in the case of longer pulses [11]. Theoretical analysis also shows that during the electric pulse, the number of large pores decreases with time; however the distribution of their radii moves toward larger values. For example, the average radius of the large pores was about two times larger at 1 ms than at 100 μs (about 20 and 10 nm respectively) [12,23].

In the majority of experimental studies the size of the pores is estimated for the pulses of a single duration [15,21] or a few different durations but in the same duration range—microseconds [24] or milliseconds [20]. There are also studies in which the relationships between various parameters, such as the amplitude, duration and/or number of pulses, of the electric treatment required for electropermeabilization—increase of the membrane permeability to the particular tracer molecules (or ions), are determined [16,18,25,26]. But in some of these studies this has been done either just for a single molecule, e.g., phenosafranine ($m = 322.8$ Da) [26], or for several molecules but rather large ones (with the molecular mass starting from several hundred, e.g. propidium iodide ($m = 668.4$ Da) [16,18], or trypan blue ($m = 891.8$ Da) [16] to tens or hundreds of

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thousands of daltons, e.g. dextrans with the average molecular mass of 10–156 kDa [16,18,20], fluoresceinated bovine serum albumin (m = 67 kDa, r = 3.5 nm) [20].

Meanwhile, it is known that the threshold of electroporation is associated with the appearance of small pores [15,21,27,28], sometimes, just a few ones [15,27]. These pores are permeable to small ions (Na^+, K^+, Rb^+, Cl^-) but are small enough to prevent slightly larger molecules from entering the cell. For example, the exposure of human erythrocytes to an exponential electric pulse with the amplitude of 3 kV/cm led to the poration of more than 80% of the cells, but the pores permeable to sucrose appeared only in 5.5% of the cells [29]. Electric field pulses of nanosecond duration (60 ns, 12 kV/cm) created pores permeable to Cl^- and alkali metal cations, but not to propidium iodide [28].

In some studies, the sizes of the pores created by a series of pulses (3–10 pulses) are estimated [16,20], which complicates the prediction of a consequence of a single-pulse exposure. Meanwhile, the majority of theoretical modeling studies are run for a single-pulse protocol [11,12,23]. So, there is a lack in the studies in which the pore sizes would be estimated for different pulse durations, especially for the amplitudes close to the threshold of electroporation.

Here, we compared the size of the pores created by square-wave electric pulses with the duration of 100 μs and 2 ms. This was done by determining the following: i) the fraction of electroporated cells (the cells the membranes of which have become permeable to small potassium ions (molecular mass m = 39 Da, $r \approx 0.16$–0.22 nm [30])); ii) the fraction of cells the membrane of which become permeable to the molecules of mannitol (m = 182.172 Da, r = 0.36–0.42 nm [21,31]), sucrose (m = 342.3 Da, r = 0.44–0.52 nm [21,31]), and bleomycin ($m \approx 1450$ Da, $r \approx 0.8$ nm); iii) the fraction of the cells that were killed by the electric treatment. In addition, the kinetics of the disappearance of the pores permeable to the molecules of bleomycin was compared for the cells electroporated by the pulses with different durations (100 μs and 2 ms).

This is the first study in which the sizes of the pores corresponding to the threshold of electroporation have been estimated for the pulses with the durations within the range of hundreds of microseconds and several milliseconds. Preliminary results were published elsewhere [32].

2. Materials and methods

2.1. Electroporation, growth and other media

The culture medium was Dulbecco's modified Eagle's medium (cat. no. D5546, Sigma-Aldrich Chemie, Steinheim, Germany) supplemented with 10% fetal bovine serum (cat. no. F7524, Sigma-Aldrich Chemie), 1% L-glutamine (cat. no. G7513, Sigma-Aldrich Chemie), 100 U/ml penicillin, and 90 μg/ml streptomycin (cat. no. P0781, Sigma-Aldrich Chemie). Electroporation was carried out on mouse hepatoma MH-22A and Chinese hamster ovary cells suspended either in the culture medium (for the determination of cell electroporation) or minimum essential medium Eagle (Sigma-Aldrich Chemie). Bleomycin hydrochloride (Bleocin, Nippon Kayaku, Tokyo, Japan) was obtained as a crystalline powder and dissolved in sterile 0.9% NaCl solution (Balkanpharma-Troyan, Troyan, Bulgaria) at a concentration of 1 mM. Further dilutions were also made in sterile 0.9% NaCl solution.

As a medium for electroporation of human erythrocytes, a 9:1 mixture of 154 mM sodium chloride and isotonic (272 mM) sucrose solution was used. For the determination of the fraction of erythrocytes with the pores permeable to mannitol and sucrose, the 9:1 mixtures of 154 mM sodium chloride and 285 mM mannitol or 272 mM sucrose solutions were used [15].

Calibration solutions containing 100 nM–100 mM KCl were prepared by diluting a stock solution of 100 mM KCl and adding 150 mM sodium chloride and 8 mM sodium benzoate [33,34]. The NaCl is added to keep sodium ion concentration close to that in the electroporation medium.

2.2. Cells

Experiments were performed with mouse hepatoma cell line MH-22A and Chinese hamster ovary (CHO), as well as human erythrocytes.

Blood was collected on heparin and the erythrocytes were isolated by centrifugation. The plasma and buffer coat comprising the white blood cells and platelets was carefully removed by aspiration. The cells were then washed three times with isotonic sodium chloride solution (0.9% NaCl) and suspended in the electroporation medium at a volume concentration of 1%. The erythrocytes were electroporated within a day of preparation.

The MH-22A and CHO cells were grown in monolayer cultures in 25-cm^2 (60–70 ml) or 75-cm^2 (250 ml) flasks (Greiner Bio-One, Frickenhausen, Germany) at 37 °C in a humidified 5% CO_2/95% O_2 atmosphere in incubator IR AutoFlow NU-2500E (NuAire, Plymouth, MN, USA). All manipulations that required sterile conditions were done in a vertical laminar flow cabinet Aura Vertical SD4 (BIOAIR Instruments, Siziano, Italy).

When cells reached confluence they were trypsinized for 2–10 min with 2 ml of 0.25% trypsin–0.02% ethylenediaminetetraacetic acid (EDTA) solution (cat. no. T4049, Sigma-Aldrich Chemie). When cells detached from the flask bottom, cell suspension was supplemented with 2 ml culture medium to protect cells from further action of trypsin. After centrifugation of the suspension for 5 min at 1000 rpm at room temperature, cells were resuspended in the culture medium at approximately $2–5 \times 10^7$ cells/ml (for the determination of the fraction of electroporated cells) or in a minimum essential medium Eagle at a concentration of $0.8–1.2 \times 10^6$ cells/ml (for the determination of the fraction of cells permeable to bleomycin and killed cells).

When the fraction of electroporated cells was determined, the MH-22A or Chinese hamster ovary cells were kept for 60–70 min at room temperature (20–21 °C) before electroporation. During this time, the cells restore the normal level of the intracellular concentration of potassium ions [34]. Then the cells were electroporated within 15–20 min. When electropermeabilization to bleomycin was studied, the cells were electroporated within 20–40 min.

2.3. Electroporation

Single square-wave pulses with the durations of 100 μs and 2 ms and the amplitudes ranging from 0.2 to 2.4 kV/cm were used. A 50-μl droplet of cell suspension was placed between a pair of flat stainless-steel electrodes and subjected to a single square-wave electric pulse. The amplitude of an electric pulse was monitored by an analog storage oscilloscope S8-13 (Russia). The distance between the electrodes was equal to 2 mm.

When determining the fraction of electroporated cells, after the exposure to an electric pulse, the cell suspension was pipetted out of the chamber, immediately transferred to a chilled Eppendorf tube, kept on ice for 5–10 min, and then kept for 30–40 min at 10–11 °C to prevent pores from closing and to allow equilibration between intracellular and extracellular K^+ concentrations. Then, the extracellular potassium concentration was measured by means of a mini K^+-selective electrode [33,34].

When determining the fraction of cells the membranes of which have become permeable to bleomycin, 45 μl of the cell suspension (prepared in a minimum essential medium Eagle) was mixed with 5 μl of 200 nM bleomycin solution, placed between two stainless-steel electrodes 2 mm apart, and subjected to a square-wave electric pulse [34].